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Effect of Dosage and Vaccination Route on Transmission of a Live Attenuated *Mycoplasma gallisepticum* Vaccine: A Broiler Model

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SUMMARY. *Mycoplasma gallisepticum* (MG) is an economically significant pathogen of poultry species. Among the table egg sector of the poultry industry, live attenuated strains of MG are commonly used to limit production losses associated with MG-induced disease. These vaccines, however, may be problematic to broiler- and turkey-related industries because of associated virulence; therefore, an understanding of the transmissibility of the live MG vaccines is of particular importance. In the present study, a broiler model addresses the effect of vaccine application route and dosage on the transmission of the MG vaccine FVAX-MG® to commingled unvaccinated subjects for 7 wk postvaccination. Vaccinations occurred at 2 wk of age via eyedrop or spray application at 1× (4×10^6 colony-forming units [cfu]), 10^{-3}× (4×10^3 cfu), or 10^{-6}× (4 cfu) of the manufacturer's recommended dosage, and subsequent transmission to unvaccinated subjects was measured. The serologic response to MG antigen and the presence of MG DNA indicated FVAX-MG transmission only within the 1× FVAX-MG eyedrop treatment. Among no other treatment was transmission of FVAX-MG detected. The results of the present study demonstrate that the dosage and vaccination route may have direct implications on subsequent transmission of FVAX-MG.

RESUMEN. Efecto de la dosis y la ruta de vacunación en la transmisión de una vacuna viva atenuada de *Mycoplasma gallisepticum*: Un modelo en pollos de engorde.

Mycoplasma gallisepticum es un agente patógeno con repercusión económica importante en las especies avícolas. Dentro de la industria avícola, en el sector de los productores de huevo para plato se utilizan comúnmente cepas vivas atenuadas de *M. gallisepticum* para limitar las pérdidas en la producción asociadas con la enfermedad producida por este agente. Sin embargo, estas vacunas pueden ser un problema para las industrias de pollo de engorde y pavos, debido a su virulencia asociada. Por lo tanto, el comprender la transmisión de las vacunas vivas de *M. gallisepticum* tiene una importancia particular. En el presente estudio, se utilizó un modelo en pollo de engorde para determinar el efecto de la ruta de aplicación y de la dosis de la vacuna en la transmisión por cohabitación a aves no vacunadas durante siete semanas después de la aplicación de la vacuna FVAX-MG® de *M. gallisepticum*. La vacunación se realizó a las dos semanas de edad por gota en el ojo o por aplicación por aerosol con concentraciones de 1× (4×10^6 unidades formadoras de colonias, UFC), 10^{-3}× (4×10^3 UFC), ó 10^{-6}× (4 UFC) de la dosis recomendada por el fabricante, y se determinó la subsecuente transmisión a las aves no vacunadas. La respuesta serológica al antígeno de *M. gallisepticum* y la presencia de ADN indicaron que ocurrió transmisión de este micoplasma únicamente con el tratamiento por gota en el ojo con la concentración 1× de la vacuna FVAX-MG. No se detectó transmisión del *M. gallisepticum* de la vacuna FVAX-MG con otros tratamientos. Estos resultados demuestran que la dosis y la ruta de vacunación pueden tener implicaciones directas en la transmisión subsecuente del *M. gallisepticum* de la vacuna FVAX-MG.

Key words: *Mycoplasma gallisepticum*, vaccine strain, transmission, vaccine administration

Abbreviations: cfu = colony-forming units; doa = days of age; MG = *Mycoplasma gallisepticum*; PBS = phosphate-buffered saline; PV = postvaccination; SPA = serum plate agglutination; woa = weeks of age

Mycoplasma gallisepticum (MG) is a major pathogen of avian species (27) and the most economically important *Mycoplasma* species impacting poultry (19,22). MG is a causative agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys and is readily transmitted by direct or indirect means. Consequences of MG infections include increased mortality and carcass condemnation and reduced egg production, hatchability, and feed efficiency (25). Economic losses are also realized through costs associated with medication, prevention, and control programs (16). Traditional control strategies for MG primarily have relied upon strict biosecurity and biosurveillance practices. Although these means have minimized MG outbreaks in the past, changes within the poultry industry, including multiage production sites and geographic proximity of various poultry-related facilities, necessitate further MG control strategies (11,14).

Within the table egg sector of the poultry industry, live attenuated MG vaccines offer an additional means of control (15). Currently, there are three live MG vaccines commercially available: F strain (FVAX-MG®, Fort Dodge Animal Health, Princeton, NJ), 6/85 (Mycovac-L®, Schering-Plough Animal Health, Omaha, NE), and ts-ll (Mycoplasma Gallisepticum Vaccine®, Merial Select, Gainesville, GA). Each has demonstrated protection against MG; however, individual characteristics (e.g., pathogenicity, virulence, transmissibility) of the vaccines vary considerably. Strains 6/85 and ts-11 are safer due to reduced pathogenicity and transmissibility (14,18), but the F strain has been shown to be more protective against MG challenge (1). The F strain vaccine reduces egg production losses due to MG infection (3,6), has demonstrated virulent MG strain displacement (13), and persists for the life of the vaccinated layer (12). Consequently, F strain is a widely used attenuated live MG vaccine (1,3,8). However, use of this vaccine may be limited due to pathogenicity to turkeys (17,20) and young broilers (26) and geographical use restrictions (15).

In lieu of the development of a safer and more effective means of control, research is necessary to fully characterize or optimize currently

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available MG vaccines. Furthermore, with the continued growth of the poultry industry and the increasing proximity of poultry facilities to each other, risks to neighboring poultry facilities should be assessed. Using a broiler model, the present study examines the effect of dosage and application route on the transmission of the commercially available live MG vaccine FVAX-MG.

MATERIALS AND METHODS

Animals and housing. Ross 708 broiler chickens ($n = 96$, unsexed) were obtained at 1 day of age (doa) from a commercial source certified MG- and *Mycoplasma synoviae* (MS)-free under National Poultry Improvement Plan (24) guidelines and placed on fresh pine (*Pinus* spp.) shavings in a conventional poultry house. At 7 doa, they were transferred and randomly allocated into 16 biological isolation units (six birds/unit) within an environmentally controlled disease isolation facility (2). Diets were formulated to meet or exceed National Research Council Guidelines (24). Feed and water were provided *ad libitum*, temperature was maintained at 25–30 C, and lighting was provided for 20 hr/day. At 8 doa, 10% of the subjects ($n = 10$) were randomly selected, bled via the wing vein, and assayed for MG and MS antibodies by serum plate agglutination (SPA) assays (28). Research subjects were maintained within guidelines approved by the Institutional Animal Care and Use Committee and were in accordance with the International Guiding Principles for Biomedical Research Involving Animals (4).

Vaccine/vaccination protocols. Sham and vaccine treatments were applied at 2 wk of age (woa). The MG vaccine FVAX-MG (Fort Dodge Animal Health) was obtained via commercial source and resuspended in sterile phosphate-buffered saline (PBS) to a concentration of 1 dose/20 μl. All serial dilutions ($10^{-3}\times$ and $10^{-6}\times$) were performed in sterile PBS (4 C), and the resulting solutions were chilled through application. For titer determination, aliquots were removed from appropriate dilutions and plated on Frey's medium containing 12% swine serum (regular) (9) and 3.5% yeast extract (Invitrogen, Frederick, MD). For eyedrop application, 20 μl of the appropriate dilution or of PBS only (sham) was administered directly to one eye of each subject via pipette. For spray application, the appropriate dosage was diluted in 30 ml of sterile PBS and administered via a coarse spray (as recommended by the manufacturer) via an Atomist™ (Root-Lowell Corp., Lowell, MI) sprayer to confined subjects at a rate of six birds per treatment. Chickens were housed within biological isolation units according to treatment. Unvaccinated subjects were maintained within four separate biological isolation units. At 4 woa, three subjects from each respective treatment were commingled with three unvaccinated subjects in each biological isolation unit.

Experimental design. Each biological isolation unit ($n = 16$) was assigned to one of eight treatments with two replicates per treatment. Treatment designation and handling procedures were designed to minimize cross-contamination among the various treatment groups. Dilutions were based on the manufacturer's recommended dosage (1×).

Treatment 1. Sham-inoculated controls—2 woa—eyedrop application.

Treatment 2. FVAX-MG ($10^{-6}\times$)-inoculated controls—2 woa—eyedrop application.

Treatment 3. FVAX-MG ($10^{-3}\times$)-inoculated controls—2 woa—eyedrop application.

Treatment 4. FVAX-MG (1×)-inoculated controls—2 woa—eyedrop application.

Treatment 5. Sham-inoculated controls—2 woa—spray application.

Treatment 6. FVAX-MG ($10^{-6}\times$)-inoculated controls—2 woa—spray application.

Treatment 7. FVAX-MG ($10^{-3}\times$)-inoculated controls—2 woa—spray application.

Treatment 8. FVAX-MG (1×)-inoculated controls—2 woa—spray application.

MG diagnostics. Subjects vaccinated via eyedrop or by spray were bled weekly from the cutanea ulnaris (wing) vein from 3 to 7 wk postvaccination (PV) (5–9 woa). To minimize the handling and the risk of vectoring the vaccine, the commonly housed unvaccinated subjects were bled at 7 wk PV only. Serum components were separated by sedimentation of the red blood cells, and failure of separation resulted in sample exclusion. All sera were tested for antibodies to MG by SPA analysis adapted from Yoder (28) using a 0 (no agglutination) to 4 (strong agglutination) scale as described by Evans *et al.* (5). Commercial antigens A and B from two sources (Charles River Laboratories, Inc., Wilmington, MA and Intervet/Schering Plough Animal Health, Millsboro, DE, respectively) were used for confirmatory purposes. Commercial antigen A was applied to SPA analyses from 3 to 7 wk PV and commercial antigen B was also applied to 7-wk-PV sera. To reduce the possibility of nonspecific reactions, low-grade responses (SPA score <2) were discarded, and only median- to high-grade responses (SPA score ≥ 2.0) were reported as positive seroconversions.

For PCR analysis, choanal cleft swabs of all subjects were collected. In brief, a rayon-tipped swab was wetted with sterile PBS and after sampling, the swabs were placed in 500 μl of sterile PBS and swirled to dislodge bound material. DNA extraction procedures were adapted from the National Poultry Improvement Plan (23)- and World Organization for Animal Health (25)-approved protocols. In brief, cell pellets were resuspended in 50 μl sterile distilled H_2O and boiled (100 C for 10 min). Extracts were stored on ice (10 min), centrifuged (14,000 × *g* for 5 min at 4 C), and resulting supernatants were stored at −20 C for subsequent PCR analyses.

Conventional PCR analyses were performed using the *mgc*2 primer set of Garcia *et al.* (10). Reactions were performed using an iCycler® thermocycler (Bio-Rad Laboratories, Hercules, CA). Each 50-μl conventional PCR reaction contained 0.2 mM of each dNTP (Sigma-Aldrich, St. Louis, MO), 1× Green GoTaq® reaction buffer (Promega, Madison, WI), 0.5 μM of each primer, 1.0 μl (1.25 U) of GoTaq DNA polymerase (Promega), and 1 μl of template solution. The amplification reaction included an initial denaturization step (95 C for 5 min) followed by a three-step cycle (45 cycles) including a denaturization step (95 C for 30 sec), an annealing step (58 C for 30 sec), and an extension step (72 C for 1 min), and the reaction was completed with a final extension step (72 C for 5 min). PCR products were visualized via gel electrophoresis in a 1.5% agarose gel containing GelStar nucleic acid stain (Lonza Rockland, Inc., Rockland, ME). Product sizes were determined by comparison to a 100-bp molecular weight ladder (Promega). Conventional PCR assay sensitivity was estimated by calculating the molecular mass of the MG strain R_{low} genome and applying standards of known concentration for the reactions. No template controls were included with each reaction, and all PCR assays were performed in duplicate.

RESULTS

Serum antibodies to MG were not detected at any level via SPA analyses from a random sampling of 10% of study-associated subjects before study initiation (8 doa). Titer determination indicated an original titer of 2.0×10^8 cfu/ml, which yielded dosages of 4×10^6 cfu, 4×10^3 cfu, and 4 cfu for the 1×, $10^{-3}\times$, and $10^{-6}\times$ FVAX-MG dosages, respectively. Serologic conversion did not differ with the commercial source of antigen as tested at 7 wk PV, confirming results associated with commercial antigen A (commercial antigen B data not shown). SPA assay of commingled subjects at 7 wk PV demonstrated seroconversion solely among the subjects placed in direct contact with the 1× FVAX-MG eyedrop-treated birds (Table 1). Among the vaccinated subjects, sham-inoculated controls vaccinated via eyedrop failed to seroconvert 3, 4, 5, 6, or 7 wk PV (Table 2). Subjects vaccinated via eyedrop at the $10^{-6}\times$ dilution also failed to seroconvert to study-defined parameters (Table 2). Likewise, eyedrop-vaccinated subjects at the

Table 1. Commingled unvaccinated subjects. Serum-plate agglutination and DNA-based assay of unvaccinated broiler-type chickens commingled with FVAX-MG-vaccinated broiler-type chickens, eyedrop *vs.* spray application.

			7 wk Postvaccination	
Vaccination route	Treatment	Replicate	SPA	Competitive PCR
Eyedrop	Sham	A	0/3[A]	0/3
		B	0/3	0/3
	$10^{-6}\times$	A	0/2[C]	0/2[C]
		B	0/2[C]	0/2[C]
	$10^{-3}\times$	A	0/2[C]	0/2[C]
		B	0/3	0/3
	1×	A	1/1[BC]	2/2[C]
		B	2/2[C]	2/2[C]
Spray	Sham	A	0/3	0/3
		B	0/3	0/3
	$10^{-6}\times$	A	0/3	0/3
		B	0/3	0/3
	$10^{-3}\times$	A	0/3	0/3
		B	0/3	0/3
	1×	A	0/3	0/3
		B	0/3	0/3

[A]Positive samples/samples tested.
[B]Serum sample unavailable due to nonseparation.
[C]Sample (serum, DNA) unavailable due to mortality.

$10^{-6}\times$ and $10^{-3}\times$ dilution of FVAX-MG failed to demonstrate seroconversion beyond a low-grade response (Table 2). Subjects vaccinated via eyedrop at the 1× dosage of FVAX-MG, however, demonstrated 100% seroconversion at 3, 4, 5, 6, and 7 wk PV. For spray-vaccinated treatments, vaccination at all levels, including sham-inoculated controls, failed to result in detectable seroconversion at any sampling from vaccinated (Table 2) or unvaccinated commingled (Table 1) subjects.

The presence of MG was determined by conventional *mgc*2 PCR (10) of choanal cleft/palatine fissure swabs collected 7 wk PV, and the sensitivity of the reaction was estimated at 105 genome equivalents. Among the eyedrop treatments, only the unvaccinated subjects commingled 2 wk PV with the 1× FVAX-MG treatment were positive via *mgc*2 PCR; and within this treatment, all of the commingled birds (replicates A and B) demonstrated detectable MG DNA (Table 1). Among other eyedrop-vaccinated treatments, no MG DNA was detected from sham-inoculated subjects or from subjects vaccinated at the $10^{-3}\times$ dilution (Table 2). However, a single bird within a single replicate of the $10^{-6}\times$ FVAX-MG-vaccinated treatment was positive for MG DNA. Within the 1× FVAX-MG eyedrop treatment, results varied within replicates because no MG DNA was associated with samples originating from replicate A, but MG DNA was detected in two of three subjects from replicate B. Among spray-vaccinated treatments, MG DNA was not detected from any commingled subject (Table 1) nor from any vaccinated subject, including sham-vaccinated controls (Table 2).

DISCUSSION

Although the F strain (FVAX-MG) has been demonstrated to effectively protect layer hens from losses associated with virulent MG challenge, the potential of this strain to elicit disease in turkeys and broilers has limited the application of this live attenuated vaccine, especially when these two sectors of the poultry industry are in proximity to layer chickens. Therefore, assessing the transmissibility of this strain is of particular importance. Kleven (12) first addressed the transmissibility of F strain, wherein leghorn pullets were vaccinated via eyedrop with 20 μl of an actively growing F strain culture (titer = 8×10^8 cfu/ml) before placement among unvaccinated leghorn pullets or adjacent to unvaccinated commercial broilers. The study demonstrated that F strain transmission readily occurred from vaccinated to unvaccinated pen mates within 4 wk PV and slowed thereafter, whereas transmission to commonly housed but nonadjacent birds was not apparent (12). Although the study clearly demonstrated the potential for F strain transmission, further work is necessary to assess the transmissibility of the commercial source (FVAX-MG) and the effects of varying dosage rates on the transmission of the vaccine. Furthermore, although eyedrop vaccinations with the F strain are still performed, application via spray is the preferred route of vaccination with the commercial source (Fort Dodge Animal Health), and the dynamics

Table 2. Vaccinated subjects. Serum-plate agglutination and DNA-based assay of FVAX-MG-vaccinated broiler-type chickens commingled with unvaccinated broiler-type chickens, eyedrop *vs.* spray application.

			Week postvaccination					
			3	4	5	6	7	
Vaccination route	Treatment	Replicate	SPA	SPA	SPA	SPA	SPA	Competitive PCR
Eyedrop	Sham	A	0/3[A]	0/3	0/3	0/3	0/3	0/3
		B	0/2[B]	0/3	0/3	0/3	0/3	0/3
	$10^{-6}\times$	A	0/3	0/3	0/3	0/3	0/3	1/3
		B	0/3	0/2[B]	0/3	0/3	0/3	0/3
	$10^{-3}\times$	A	0/2[B]	0/2[B]	0/3	0/2[B]	0/3	0/3
		B	0/2[B]	0/3	0/3	0/3	0/3	0/3
	1×	A	1/1[B]	3/3	3/3	3/3	2/2[B]	0/3
		B	1/1[B]	3/3	3/3	3/3	3/3	2/3
Spray	Sham	A	0/3	0/3	0/3	0/3	0/3	0/3
		B	0/3	0/3	0/3	0/3	0/3	0/3
	$10^{-6}\times$	A	0/3	0/3	0/3	0/3	0/3	0/3
		B	0/2[B]	0/1[B]	0/3	0/3	0/3	0/3
	$10^{-3}\times$	A	0/3	0/3	0/3	0/3	0/3	0/3
		B	0/3	0/3	0/3	0/3	0/3	0/3
	1×	A	0/2[B]	0/3	0/3	0/3	0/3	0/3
		B	0/3	0/3	0/3	0/3	0/2[B]	0/3

[A]Positive samples/samples tested.
[B]Serum sample unavailable due to nonseparation.

of this application route on subsequent transmission has not been addressed.

In the present study, transmission of FVAX-MG as evidenced by serologic response or by DNA-based detection was only observed among subjects commingled with the 1× FVAX-MG eyedrop-vaccinated subjects. These findings are in agreement with those of Kleven (12) who applied a similar eyedrop dosage (8×10^8 cfu/ml) as that of the present study (2.0×10^8 cfu/ml) but used a distinct source of inocula (active F strain culture *vs.* commercially available FVAX-MG) to demonstrate transmission. Within the present study, only subjects vaccinated via eyedrop with 1× FVAX-MG exhibited seroconversion above a low-grade response and within this treatment, seroconversion occurred among all vaccinated subjects. Also, only subjects from this group and a single bird from the 10^{-6}× FVAX-MG treatment yielded detectable MG DNA. It is interesting that the serologic and DNA-based assays were not in 100% agreement, but factors including assay sensitivity (estimated at 105 genome equivalents), stage of infection, and isolation protocols may have limited the DNA-based findings.

Among the unvaccinated subjects commingled with spray-vaccinated subjects, there was no indication FVAX-MG transmission. In addition, among the spray-vaccinated treatments, no vaccinated subject demonstrated either a serologic response or the presence of MG DNA. This finding was unexpected because previous research has demonstrated seroconversion and vaccine persistence in association with F strain spray vaccination of broilers up to 7 wk PV (26). However, the study used inocula distinct from that of the present study (live F strain culture *vs.* lyophilized FVAX-MG) and may explain differences in research findings.

Within the present study, the lack of detectable MG-DNA and serologic conversions associated with those birds vaccinated via eyedrop at levels less than 1× (4×10^6 cfu) and all birds spray vaccinated are indicative of limited *in vivo* FVAX-MG populations that could have limited subsequent transmission. Feberwee *et al.* (7) demonstrated a direct relationship between MG inoculation loads and the resulting *in vivo* MG populations. In the present study, eyedrop inoculations at the 10^{-3}× or 10^{-6}× FVAX-MG dosages or spray inoculations may have resulted in limited populations of FVAX-MG and undetectable serologic responses within the time constraints of the study. Furthermore, MG transmission has been directly related to the phase of the infection (21). Therefore, it is highly probable that given sufficient time, all vaccinated birds within the present study would have generated detectable serologic responses and populations of FVAX-MG. However, the ultimate transmissibility of these populations is only speculative, and further research is required to fully describe this aspect as it relates to low-dosed poultry.

Due to the importance of FVAX-MG transmissibility, factors impacting transmissibility should be characterized. The results of the present study indicate that the dosage and vaccination route may have direct implications on subsequent transmission of FVAX-MG.

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